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Review

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PSMA specific antibodies and their diagnostic and therapeutic use

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Prostate-specific membrane antigen (PSMA) is a membrane-bound glycoprotein highly restricted to prostatic epithelial cells. PSMA expression is increased in association with prostatic cancer, particularly in hormone refractory disease. Given its membrane-bound character, PSMA is an ideal sentinel molecule for use in targeting prostatic cancer cells. Monoclonal antibodies specific for PSMA are available, beginning with the antibody 7E11.C5 which originally defined PSMA and which has been developed for use in cancer detection *via* immunoscintiscanning in the ProstaScint™ test. Newer second generation antibodies specific for both linear amino acid sequence epitopes and protein conformational epitopes on the extracellular domain of PSMA have been reported. Although most of these are murine antibodies, both humanised and fully human examples have been developed. These antibodies are beginning to work their way into clinical applications for potential improved diagnostic and therapeutic uses. Results to date suggest that antibodies specific for extracellular epitopes are significantly better for clinical uses *in vivo* than the 7E11.C5 antibody that is specific for an intracellular epitope. Current knowledge relating to PSMA-specific antibodies and their clinical uses and potential is described and evaluated.

Keywords: antibody therapy, linear sequence epitopes, monoclonal antibodies, prostate cancer, prostate-specific membrane antigen, PSMA, protein conformational epitopes

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1. Introduction

PSMA is a highly glycosylated 750 amino acid type II transmembrane protein expressed on the surface of prostatic epithelial cells. It is composed of three distinct domains, a 19 amino acid intracellular, N-terminal domain, a 24 amino acid transmembrane domain and a 707 amino acid extracellular domain [1]. Although the amino acid composition of PSMA indicates it has a protein molecular weight of approximately 84,000, PSMA migrates in an SDS gel with an apparent molecular weight of 110 - 120 kDa through use of ten potential N-linked glycosylation sites which occur in the extracellular domain of the protein [2].

PSMA was identified using the monoclonal antibody 7E11.C5 [3] and has been shown to be highly prostate specific [3-9]. Quantitative expression data indicates that, at protein level, significant PSMA expression is confined to the prostate [10]. Weak extraprostatic expression of PSMA has been detected in other human tissues such as brain, salivary gland and small intestine tissues by genetic, immunohistochemical and Western blot results

[3-10]. Other results demonstrate that PSMA expression is increased in prostatic cancer, particularly in association with disease progression and in hormone refractory tumours [4,11,12].

High expression of PSMA in normal and malignant prostatic tissues and its nature as an integral membrane protein are properties which make PSMA particularly useful for antibody targeting in cell-based diagnostic or therapeutic strategies. This especially applies to antibodies directed against the extracellular domain of the protein. Multiple investigators have reported the isolation of second generation antibodies specific for extracellular epitopes on PSMA. The purpose of this review is to summarise what is known about these antibodies, their current clinical utility and discuss which properties are optimally suited for *in vivo* clinical applications in humans.

2. PSMA specific monoclonal antibodies

2.1 7E11.C5

The 7E11.C5 antibody was developed in 1987 by Horoszewicz *et al.* by immunising mice with human prostatic cancer LNCaP cells [3]. Resulting hybridomas were screened for their reactivity with LNCaP cells and negative reactivity with 32 other human non-prostatic normal and malignant cell lines. Immunohistochemical results reported by Horoszewicz *et al.* demonstrated nearly complete 7E11.C5 antibody binding specificity to epithelial cells of the human prostate [3]. This staining pattern has been largely confirmed in subsequent studies with weak extraprostatic expression of PSMA observed in brain, salivary gland and small intestine tissues [6,8]. PSMA, as defined by the 7E11.C5 antibody, has been shown to be a superior immunohistochemical marker than prostate-specific antigen (PSA) for prostatic epithelial cells [13,14]. In addition, staining of vascular endothelium of non-prostatic tumours has been reported using the 7E11.C5 antibody [8]. As discussed below, this result has been confirmed in other studies which conclude that PSMA expression is characteristic of tumour-associated neovasculation but is absent in normal vascular endothelium [15,16].

The high specificity of the 7E11.C5 antibody for prostatic epithelial cells and the overexpression of PSMA in prostatic cancer formed the basis for use of this antibody in the ProstaScint™ test for *in vivo* diagnostic imaging of occult prostate cancer. Studies have demonstrated that the 7E11.C5 antibody is

specific for a linear protein sequence epitope composed of the first six amino acids from the N-terminal of the PSMA protein. This portion of the protein is distributed on the cytoplasmic side of the plasma membrane [17]. This places the epitope in an isolated environment in live prostatic epithelial cells. Thus, the 7E11.C5 antibody is most probably detecting non-viable or apoptotic cells. To accumulate an adequate quantity of such cells or cellular debris in tumours for detection, a minimum tumour size is likely to be required. This physical restriction is likely to limit the potential of this antibody for *in vivo* diagnostic or therapeutic applications and most probably also limits the clinical performance of the ProstaScint™ imaging agent.

2.2 Antipeptide antibodies

The identification of the intracellular distribution of the 7E11.C5 antibody epitope has helped focus efforts by multiple groups in generating monoclonal antibodies specific for extracellular epitopes on PSMA. Antibody production efforts have utilised full length protein, fragments of the PSMA protein and short peptides composed of 6 - 8 amino acids of the PSMA sequence selected for their probability of comprising an antigenic determinant by use of, for example, the Hopp and Woods test [18].

Murphy *et al.* reported the first monoclonal antibody specific for an extracellular epitope on PSMA [19]. This IgM antibody, 3F5.4G6, was obtained by immunising mice with a peptide corresponding to aa716-723 of the PSMA protein which is distributed near the C-terminal of the protein. Results demonstrated that the 3F5.4G6 antibody was capable of immunoprecipitating native PSMA from an LNCaP cell detergent lysate. The immunoprecipitated protein was reactive with the PSMA-specific 7E11.C5 antibody in a subsequent Western blot. The immunoreactivity of this peptide region was further demonstrated in another report in which animals were immunised with a crude membrane preparation from a prostatic carcinoma and an antibody, designated 4G5, was screened out using the aa716-723 peptide [20]. This result also confirms that the aa716-723 peptide epitope is exposed and accessible in the native protein and its conformation in the native protein is most probably similar to that found in the isolated peptide.

Another application of this approach has involved generation of rabbit polyclonal antiserum using other

potential antigenic determinants within PSMA (Holmes, unpublished results). For example, rabbits immunised with KLH-peptide conjugates with aa63-68, aa132-137, or aa482-487 peptides in each case generated high antipeptide specific immunoreactivities. However, no binding to full length PSMA could be demonstrated with these antisera under conditions that gave saturating antipeptide reactivity. Presumably, expression of these epitopes in the context of the protein was important and affected the antibody binding ability. This observation may be a property of antibodies to these protein regions, or perhaps can be overcome by the use of slightly longer eight amino acid peptides as was utilised by Murphy *et al.* [19].

2.3. Linear epitope specific antibodies

Multiple groups have reported other anti-PSMA monoclonal antibodies specific for linear amino acid sequence epitopes distributed throughout the extracellular domain [10,15,21,27-30]. In general, these antibodies have been derived by immunisation of animals with full length PSMA either in membranes or as purified protein. Thus, although the epitopes recognised by these antibodies are of the same type as those recognised by antipeptide antibodies, their location in the protein sequence is not precisely known.

Liu *et al.* reported the isolation of four hybridomas, each secreting an antibody specific for the extracellular domain of PSMA [21]. Competitive binding studies demonstrated that the four antibodies defined two distinct, non-competing epitopes on the extracellular domain of PSMA. The antibodies were also shown to bind to viable LNCaP cells *in vitro* and showed strong immunohistochemical staining of prostatic epithelium and vascular endothelium within a wide variety of carcinomas [21]. These antibodies were shown to bind to denatured PSMA based upon their ability to detect PSMA by Western blot [21]. A subsequent report demonstrated that viable LNCaP cells internalise antibodies of this panel and that PSMA is constitutively endocytosed in LNCaP cells, a process whose rate is increased by anti-PSMA antibodies [22]. These observations suggested utility of these antibodies for targeting and delivery of toxins, drugs, or radioisotopes to the interior of prostate cancer cells.

Recent studies of binding properties of these antibodies after radiolabelling indicate competition for binding sites occurs between certain antibody

pairs suggesting at least two closely distributed but non-identical binding sites are recognised [23]. In addition, quantification of the number of sites per cell bound by the antibodies, indicated a decrease of about 40%, when comparing the number of sites detected in permeabilised LNCaP cells with viable cells. The data presented demonstrated that efficient antibody uptake by LNCaP cells occurred along with metabolic processing of the antibody. The results demonstrated that elimination of the radioisotope from the cell was dependent on the nature of the radiolabel used. That is, antibody degradation and efficient elimination of ^{131}I -label was reported, however, radiometal chelates from degraded antibody molecules were released from LNCaP cells at a significantly slower rate suggesting a much greater radiotherapeutic potential for radiometal chelates such as ^{90}Y -DOTA. In general, the results demonstrated that antibodies J415 and J591 had the highest binding affinity and were promising reagents for targeting PSMA *in vivo* [23].

Studies of immunoconjugates incorporating either cytotoxic drugs [24], ^{90}Y [25] or the α -particle emitter ^{213}Bi [26] into the J591 antibody have been conducted to test their ability to specifically kill prostatic cancer LNCaP cell tumours grown in nude mice. Immunoconjugates composed of the maytansine analogue DM1 coupled to the J591 antibody provided a reduction of tumour size in mice with minimal toxicity at a dose of 300 $\mu\text{g/day}$ [24]. Unconjugated DM1 at equimolar doses was lethal and PC3 cell tumours which do not express PSMA were unaffected by the immunoconjugate demonstrating effective antigen-specific delivery of the immunoconjugate to PSMA positive tumours with low systemic toxicity. Smith-Jones *et al.* reported that ^{90}Y -DOTA-J591 reduced the tumour volume at dose ranges between 30 - 90 μCi [25]. Re-treatment resulted in further reduction in tumour size or complete regression. Uncontrolled tumour growth occurred in mice treated with ^{90}Y -DOTA-labelled irrelevant mouse antibody [25]. ^{213}Bi -labelled J591 demonstrated effective LNCaP cell killing both *in vitro* and *in vivo* in LNCaP cell tumour bearing nude mice associated with internalisation of the radiolabelled J591 antibody [26]. Median tumour free survival times were significantly increased for animals receiving ^{213}Bi -J591 compared with a ^{213}Bi -labelled irrelevant antibody or unlabelled J591. Serum PSA levels, as secreted from the LNCaP cell tumours, was also decreased in animals receiving ^{213}Bi -J591 compared with other treatment groups.

Overall, these results suggest a potential for drug and radioimmunoconjugates incorporating antibodies specific for the extracellular domain of PSMA for therapy of prostatic cancer.

Immunohistochemical studies using these and other antibodies have been conducted to confirm PSMA expression in vascular endothelium from a variety of human non-prostatic cancers and absence of expression in normal neovasculature [15,16]. Despite staining of vascular cells in cancerous tumours, no staining of neoplastic cells from non-prostatic origins was observed. Thus, PSMA specific monoclonal antibodies may also be effective in general targeting of tumour neovasculature *in vivo*.

A panel of monoclonal antibodies specific for epitopes distributed within the extracellular domain of PSMA has been reported by Murphy *et al.* [27]. This report detailed binding specificity of monoclonal antibodies reactive with distinct regions of the PSMA extracellular domain selected from a large panel of 36 distinct linear epitope specific monoclonal antibodies [28]. From this large panel of antibodies, certain generalisations can be made. Solid-phase ELISA studies demonstrated that these antibodies bound to native full-length PSMA, as well as to denatured recombinant fusion proteins containing portions of the PSMA molecule. Efficient binding to denatured full-length PSMA by these linear epitope specific antibodies was demonstrated by their ability to detect PSMA in Western blot experiments. Moderate binding of extracellular linear epitope specific antibodies occurred to non-viable PSMA expressing cells when compared with antibody binding to intracellular epitopes by flow cytometric analysis [27]. Staining intensity by flow cytometry was generally weaker with viable cells than with non-viable or fixed cells when linear epitope specific antibodies were used.

Other anti-PSMA antibodies have been produced such as Hybritech PEQ226.5 and PM2J004.5 antibodies [15]. Both antibodies effectively stain PSMA expressed in tissues by immunohistochemistry with the PEQ226.5 antibody recognising an extracellular epitope and PM2J004.5 recognising an intracellular epitope. The PEQ226.5 antibody has been paired with 7E11.C5 in a sandwich ELISA and used to study specificity of PSMA expression at a protein level in human tissues [10]. Very high levels of PSMA were found to be present in normal and malignant prostatic tissues. Low levels of PSMA were observed in membranes from ovary and breast. Negligible levels (about 200- to 500-fold lower

than found in prostatic tissues) were found in membranes from skin, liver, large and small intestine and kidney. Thus, at protein level, quantification by sandwich ELISA indicates PSMA to be overwhelmingly prostate-specific.

Olson recently reported the isolation of two murine antibodies specific for linear epitopes in the extracellular domain of PSMA [29]. Although characterisation information is currently limited, one antibody was reported to react with both native and denatured PSMA, the other only with denatured PSMA. Finally, Wolchok reported the production of a panel of murine monoclonal antibodies from a mouse immunised with human PSMA [30]. Antibody specificity was determined by ELISA, Western blot and flow cytometry. Approximately 90% of the ELISA reactive clones recognised both human and mouse PSMA. Mouse PSMA was localised to a subset of proximal renal tubules.

2.4 Conformational epitope specific antibodies

A second class of antibodies specific for epitopes present on the extracellular domain of PSMA recognise conformational folding of the PSMA molecule. These epitopes arise from the juxtapositioning of amino acid residues from differing portions of the linear sequence in close proximity in 3-dimensional space in a manner characteristic of the native protein. Such antibodies have inherently dissimilar properties from linear epitope antibodies, most notable is their inability to bind to denatured PSMA. Tino *et al.* [28] reported the isolation of three murine hybridomas which secrete antibodies capable of binding to at least two distinct conformational epitopes on PSMA. Their results demonstrated the inability of these antibodies to bind to fragments of PSMA, or to heat-denatured full length PSMA. Additionally, these antibodies were incapable of detecting PSMA in a Western blot. In contrast, native PSMA was efficiently immunoprecipitated by this antibody panel. Most significantly, flow cytometric analysis demonstrated strong and equivalent immunostaining of both viable and non-viable PSMA expressing cells.

A panel of five fully-human monoclonal antibodies which bind to a similar conformational epitope present in the extracellular domain of PSMA have been developed using HuMAb-Mouse™ strains [31]. As with murine conformational antibodies, these human antibodies do not recognise denatured

antigen and demonstrate strong high affinity binding to either viable or fixed PSMA-expressing cells by flow cytometry. Other studies indicate specificity for prostatic epithelial cells in immunohistochemical analyses with human tissues, efficient internalisation into PSMA-expressing cells *in vitro*, capability of mediating a strong ADCC but not CDC response and efficient biodistribution to PSMA-expressing tumours in mouse model systems (Holmes, unpublished results).

It is particularly noteworthy to emphasise that in all cases so far tested, flow cytometric studies of antibodies specific for conformational epitopes of PSMA demonstrate significantly stronger staining of PSMA-expressing cells (often one log or greater staining intensity) compared with that observed with linear epitope-specific antibodies. Binding of linear epitope-specific antibodies to LNCaP cells is sensitive to the viability of the cells with substantially less binding occurring to viable cells (or as measured as a lower number of apparent sites per cell). One possible explanation for this is the presence of an intracellular pool of PSMA that is only available in non-viable or fixed cells. However, no difference in staining intensity is observed with conformational antibodies when either viable cells or non-viable unfixed or fixed cells are used. This suggests perhaps instead that an intracellular PSMA pool is not large and/or that linear epitope antibodies are able to bind to only a portion of the total cell surface PSMA molecules recognised by conformational antibodies. This could be a function of linear sequence epitope crypticity by virtue of protein folding or glycosylation effects. In contrast, protein folding effects define conformational epitopes and thus these antibodies should not be sensitive to epitope crypticity. This is an important distinction and suggests that conformational antibodies will prove to be superior targeting reagents for *in vivo* applications in humans.

Recently, the isolation of four additional murine antibodies specific for conformational epitopes on PSMA has been reported [29]. These antibodies also do not bind denatured PSMA and efficiently bind to PSMA-expressing cells. Future studies will be required to characterise the nature of the conformational epitopes recognised by these antibodies and their detailed binding properties.

3. Current clinical applications with PSMA specific antibodies

3.1 ProstaScint™ scans

Primary extraprostatic spread or failure after prostate cancer treatment can occur locally at the prostatic fossa and/or metastasise to regional and/or distant lymphatics and possibly bone [32]. Non-invasive diagnostic tools such as computed tomography (CT) or magnetic resonance imaging (MRI) are sensitive to the size of lymph nodes but cannot detect the presence of prostatic cancer cells [33]. Although not effective for bone metastases, one means to directly target lymph node metastases is the ProstaScint™ scan which is based upon use of ¹¹¹In-labeled 7E11.C5 antibody [34,35]. This immuno-scintigraphic scan recognises PSMA expression chiefly in lymph nodes and the prostate bed [36].

Multiple investigators have studied the sensitivity and specificity of the ProstaScint™ scan in prostate cancer patients who underwent conventional CT or MRI scanning, surgical or needle biopsies, or rising serum PSA after radical prostatectomy [36-47]. Pooled results from 637 ProstaScint™ scans described in these reports had a sensitivity of 73% and an accuracy of 82%. For comparison, the sensitivity of conventional CT scanning ranges from about 4 - 15% in these patient groups [33,41,47]. This is similar to the 15% sensitivity reported for MRI [47]. The ProstaScint™ scan is also useful in determining prior to treatment if prostate cancer will recur or has spread to other parts of the body [42,43]. Khan *et al.* have reported that salvage radiotherapy was more likely to lead to a durable complete PSA response in men with prostate cancer who failed radical prostatectomy and had a negative ProstaScint™ scan outside the pelvis as compared with those who had a positive scan [43]. ProstaScint™ was also more predictable than prognostic tables in predicting lymph node involvement prior to lymph-adenectomy in 198 prostate cancer patients with clinical stage T2 to T4 [43].

While no staging modality has been ideal in diagnosis of local recurrence, regional or distant soft lymph node metastases of prostate cancer, ProstaScint™ has contributed to diagnosis, complemented and often exceeded the diagnostic ability of conventional modalities [33,36-50]. Given the epitope specificity of the 7E11.C5 monoclonal antibody used in the ProstaScint™ scan [17], it is likely that a superior scan with

enhanced sensitivity that is capable of identifying smaller lesions would be possible through use of a monoclonal antibody specific for an epitope within the extracellular domain of PSMA and which efficiently binds to viable prostate cancer cells.

3.2 Clinical studies of *in vivo* antibody targeting and therapy of prostate cancer

A Phase I dose-escalation study has been conducted using ^{90}Y -labelled CYT-356 (murine antibody 7E11.C5) in patients with hormone-refractory prostate cancer that had first been imaged using ^{111}In -CYT-356 [51]. Doses of ^{90}Y ranged from 1.83 - 12 mCi/m². The maximum tolerated dose of the radioimmunoconjugate was 9 mCi/m² with myelosuppression as the dose limiting toxicity. No patients (0/12) had a complete or partial response, although 3/12 had a transient subjective improvement in their symptoms. A slightly longer period before progression in patients receiving the highest dose compared with the next lower dose was also observed. These results are consistent with the specificity of the CYT-356 (7E11.C5) antibody for an intracellular epitope of PSMA and its resultant inability to bind to viable prostatic cancer cells. A potential therapeutic effect from ^{90}Y -CYT-356 would have to come from a bystander effect wherein radiolabelled antibody would bind to non-viable or apoptotic PSMA-expressing cells within the tumour in an effort to treat the adjacent viable tumour cells. This is a severe limitation of the CYT-356 antibody. A much greater likelihood for effective treatment would be expected using antibodies specific for PSMA epitopes expressed on the outer surface of tumour cells allowing direct binding of the therapeutic antibody conjugates to viable cells for cell killing.

Clinical applications involving second-generation antibodies specific for extracellular epitopes on PSMA, although in their early stages, offer promise. The linear epitope specific murine huj591 antibody [21] and its humanised counterpart huj591 [52] are being used in Phase I biodistribution trials. Biodistribution results with the murine huj591 antibody are complete and indicate selective localisation to bone or soft tissue disease with no localisation to sites other than prostatic cancer sites [53,54]. Of 32 evaluable patients, overall tumour targeting was reported in 26/32 (81%) patients. Targeting was observed in 25/30 (83%) of patients with bone disease and 8/11 (73%) of patients with soft tissue disease. Preliminary results from a Phase I trial using the huj591 antibody with trace

labelling with ^{111}In indicate no apparent toxicity or immunogenicity of the humanised antibody [53]. Early targeting results indicate antibody uptake demonstrating targeting with excellent cancer specificity to both bone and soft tissue disease. Additional Phase I trials with ^{90}Y -labelled huj591 for treatment are planned.

3.3 Use of PSMA as a cellular immunotherapy target

In addition to antibody based applications utilising PSMA as a therapeutic target, active cellular immunotherapy for prostate cancer has been studied utilising antigen-presenting dendritic cells loaded with peptides derived from PSMA to activate a T-cell response [55]. Positive results were achieved in Phase I and II trials with two PSMA HLA-A2-restricted epitopes exogenously loaded onto dendritic cells. Approximately 30% of study participants showed treatment benefit, defined as a steady decrease in serum levels of PSA and/or stabilisation or decrease in metastatic tumour burden [56]. These results were quite encouraging, yet they could not circumvent the conceptual drawback that the immunodominant, cellular targets on the surface of prostate tumour cells are not known. In order to effectively address this concern, a Phase I/II clinical trial is currently underway involving autologous dendritic cells loaded with whole, recombinant human PSMA protein. This approach directs a significant portion of the antigen into the MHC class I processing and presentation pathway, even though it is exogenously added. Such a strategy is aimed at mounting a cellular immune response in hormone-refractory prostate cancer patients and to overcome tolerance to PSMA that, after all, is also expressed on normal prostate cells. Preliminary results suggest that both cellular and humoral responses to PSMA can be induced, regardless of the advanced age and disease state of the participants (Elgamal, unpublished results). Over 50 injections were administered intradermally with no serious adverse events, at doses from 5 - 20 x 10⁶ cells. Compared with baseline, 9/10 evaluable subjects became positive upon delayed-type hypersensitivity testing against a vaccine component. Seven of nine subjects converted to a seropositive state, as evidenced by the development of significant titres of anti-PSMA antibodies. This also suggested that tolerance was broken, at least in the realm of humoral immunity. Finally, to date, more than half of evaluable patients have displayed positive cellular immune

responses (*via* proliferation assay) to PSMA. In total, these initial data produced promising results. It is hoped that this strategy may provide additional therapeutic means to effectively treat prostate and other cancers.

4. Expert opinion and conclusion

The high prostate tissue/tumour specific expression and integral plasma membrane distribution of PSMA creates an ideal combination for its use as an *in vivo* target for antibody-based prostate cancer diagnosis and therapy. Antibody properties also are critically important for optimal results. Most attractive are specific human or humanised monoclonal antibodies that optimally bind to antigen expressed on the extracellular surface of live cells. These include antibodies that recognise both linear amino acid sequence and protein conformational epitopes present on the extracellular portion of PSMA. Linear sequence epitope antibodies are currently in use in human clinical trials and show considerable promise. Similar studies using human conformational antibodies will be conducted. *In vitro* properties of conformational antibodies suggest that they may prove to be optimal targeting reagents for clinical diagnostic and therapeutic applications. Given that few treatment options currently exist for patients with hormone refractory metastatic prostate cancer, antibody-directed therapy offers considerable potential as a stand alone therapy, or in combination with other treatment regimens including dendritic cell based cellular immunotherapy targeting PSMA expressing tumours. Antibody therapy could perhaps have its greatest impact on earlier stage patients in helping prevent recurrence of cancer, particularly in understaged patients. These new therapeutic options should lead to more effective and economical diagnosis and treatment of the disease.

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